Distribution of Adenosine-5′-Diphosphatase Activity in the Lactating Bovine Mammary Gland

ABSTRACT

A Ca^{2+}- and Mg^{2+}-stimulated adenosine-5′-diphosphatase has been found in lactating bovine mammary glands. The enzyme is associated with membranes of mitochondrial, microsomal, and Golgi apparatus fractions. The pH activity curves for the Golgi apparatus and microsomal fractions display two distinct maxima, one at pH 5.8 and the other between pH 7.4 and 8.4. Studies with activators and inhibitors indicate the enzyme is similar to adenosine-5′-diphosphatase found in other tissues. Its occurrence in the Golgi apparatus fraction indicates a possible role for this enzyme in the milk secretory process, particularly in ATP cycling in vesicles. Its occurrence in the microsomal fraction suggests a role in plasma membrane functioning.

(Key words: milk, secretion, enzymes, casein)

Abbreviation key: **ADPase** = adenosine-5′-diphosphatase, **MOPS** = 3-(N-morpholino) propane sulfonic acid, **NDPase** = nucleotide diphosphatase, **PIPES** = piperazine-N-N′-bis (2-ethane sulfonic acid), **TPP** = thiamine pyrophosphate chloride, **TPPase** = thiamine pyrophosphatase, **UDP** = uridine-5′-diphosphate, **UDPase** = uridine-5′-diphosphatase.

INTRODUCTION

Nucleotide diphosphatases in general are widespread in nature and occur in a variety of tissues (6, 8, 15). The adenosine-5′-diphosphatases (ADPase), in particular, are often found in the plasma membranes of cells, where they may function to prevent ADP-mediated interactions between cells (15). Thiamine pyrophosphatase (TPPase), another diphosphatase, is associated specifically with Golgi apparatus (21). The nucleotide diphosphatase (NDPase) activity in lactating rat mammary Golgi apparatus has been reported to be specific for uridine-5′-diphosphate (UDP) and to have little or no reactivity toward ADP (12). This manuscript deals with a study of the distribution and characterization of ADPase activity of bovine mammary gland.

MATERIALS AND METHODS

Materials

Dithiothreitol, 1-levamisole, sodium metavanadate, oligomycin (mixture of A, B, and C), ADP di(monocyclohexyl ammonium) salt from equine muscle, UDP Tris salt from yeast, malachite green hydrochloride, P^1, P^5-di(adenosine-5′) pentaphosphate sodium salt, 5′-adenylylimido-diphosphate lithium salt, ouabain, polyvinyl alcohol (soluble in cold water), and thiamine pyrophosphate chloride (TPP) were purchased from Sigma Chemical Company (St. Louis, MO). Bovine serum albumin was obtained from Pierce Chemical Company (Rockford, IL).

Mammary Glands

Whole mammary glands from cows of known good health and productivity were obtained through the cooperation of J. E. Keys and R. H. Miller of USDA, Beltsville, MD. Whole mammary glands were obtained at slaughter, trimmed to remove adipose tissue, and sectioned into pieces approximately 10 × 15 × 15 cm. The tissue was either processed

immediately or frozen on dry ice and stored at –80°C until used.

Tissue Fractionation

The procedures were carried out at 4°C unless specified. Golgi apparatus was isolated according to the method of Morré (17) with the modifications of Szymanski and Farrell (22) introduced for frozen tissue. For Golgi apparatus, mammary glands were minced and suspended in 3 vol of medium A (37.5 m*M* TRIS-maleate, mono[Tris (hydroxymethyl) amino methane] maleate, buffer, pH 6.5; 1 m*M* EDTA, 25 m*M* $MgCl_2$, and .1 m*M* dithiothreitol) containing .5 *M* sucrose and 1% dextran. They were homogenized for 1 min (position 6) using a Polytron 10 ST homogenizer (Brinkman, Westbury, NY). The homogenate was squeezed through one and then two layers of cheesecloth to remove unbroken cells and connective tissue. To concentrate the Golgi apparatus, the filtered homogenate was centrifuged at 4000 × *g* for 15 min in a swinging bucket rotor. The resulting pellets were washed once in homogenization buffer and resedimented at 4000 × *g*. The friable top one-third of the pellet was resuspended in 10 to 20 ml of medium A containing .5 *M* sucrose and 1% dextran, layered over 1.5 vol of medium A containing 1.25 *M* sucrose and 1% dextran, and then centrifuged at 100,000 × *g* for 30 min. Finally, the 1.25 *M* sucrose-homogenate interface (Golgi apparatus fraction) was removed, diluted with medium A containing .5 *M* sucrose and 1% dextran, and resedimented at 4000 × *g* for 15 min. The pellet was resuspended in medium A (2 to 3 ml), frozen, and stored at –80°C. Other subcellular fractions—including nuclei, mitochondria, microsomes, and cytosol—were isolated from the combined supernatants and pellets remaining after the recovery of Golgi apparatus as described by Bingham et al. (1). The subcellular fractions other than Golgi apparatus were diluted in medium A, but with .25 *M* sucrose, and stored at –80°C.

Protein Assay

Protein was determined using the BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Company, Rockford, IL) at 25°C using the standard protocol. Bovine serum albumin was the standard.

Enzyme Assays

The NADPH-cytochrome *c* reductase was determined by the method of Masters et al. (16). Succinic dehydrogenase and lactose synthetase were assayed according to the methods of Pennington (20) and Palmiter (19), respectively.

The Mg^{2+}-ADPase, Mg^{2+}-uridine-5′-diphosphatase (UDPase), and Mg^{2+}-TPPase were measured by the method of Chan et al. (5). Each enzyme assay contained 10.0 m*M* $MgCl_2$, 1.0 m*M* appropriate substrate, and 30 m*M* MOPS (3-[N-morpholino] propane sulfonic acid) at pH 7.4; 50 m*M* PIPES (piperazine-N-N′-bis[2-ethane sulfonic acid]) were substituted for the pH 5.8 assay.

For studies of the subcellular distribution, ADPase assays were incubated at pH 7.4 for 15 min with 45 to 55 µg of protein (cytosol at 250 µg of protein) and had zero time blanks for all subcellular fractions except Golgi apparatus. These enzymes were incubated for 25 min with about 15 µg of protein and had 10-min blanks, which were necessary for the linear range of enzyme assay. The TPPase of all subcellular fractions was incubated for 40 min with protein concentrations similar to those for ADPase and had zero time blanks. All assays were carried out within the linear range of the reactions with respect to incubation time and enzyme concentration, had a total volume of 1.0 ml, and—except where

TABLE 1. Hydrolysis of uridine-5′-diphosphate (UDP) and ADP by mammary membrane-associated enzymes.[1]

pH		Specific activity			
Activity		Golgi apparatus		Microsome	
		(nmol/min per mg)			
		$\bar{x}$	SD	$\bar{x}$	SD
5.8	UDP	19.7	7.6	19.7	5.9
	ADP	6.4	3.7	15.9	6.4
	UDP:ADP	3.08		1.24	
7.4	UDP	36.1	9.3	46.8	4.0
	ADP	31.8	1.0	24.4	4.5
	UDP:ADP	1.14		1.91	

[1]Standard assay pH 7.4; mean of three preparations.

TABLE 2. Subcellular distribution of Mg^{2+}-adenosine-5′-diphosphatase (ADPase) and thiamine pyrophosphatase (TPPase) activities in lactating bovine mammary gland.

Subcellular fraction	Specific activity					Enzyme marker ratio[2]
	ADP pH 5.8[1]		ADP pH 7.4[1]		TPP pH 7.4[2]	
	(nmol/min per mg of protein)					
	Avg	SD	Avg	SD		
Homogenate	6.7	1.6	10.4	2.3	11.2	1.00
Nuclei	5.8	3.3	13.6	1.0	13.3	. . .
Mitochondria	24.1	9.5	16.7	4.3	33.9	5.93[3]
Golgi[4]	6.4	3.7	31.8	1.0	58.2	14.4[5]
Microsomes	17.5	3.0	24.2	4.2	23.6	4.26[6]
Cytosol	4.5	1.3	10.5	3.1	16.4	. . .

[1]Average values three preparations; one fresh, two frozen.

[2]Average values two preparations; both frozen.

[3]Succinate dehydrogenase; ratio of specific activity in mitochondrial fraction to homogenate.

[4]Average values three preparations; all frozen.

[5]Lactose synthetase; ratio of specific activity in Golgi fraction to homogenate.

[6]NADPH:cytochrome *c* reductase; ratio of specific activity in microsomal fraction to homogenate.

noted—had an incubation temperature of 25°C. The blanks were used to correct for any nonspecific activity.

At the end of the incubation time, a 200-µl aliquot of the reaction mixture was added into 800 µl of the malachite green-molybdate-polyvinyl alcohol mixture (5). The inorganic phosphate released from the hydrolysis of the substrate reacts with the malachite green-molybdate-polyvinyl alcohol mixture to form a stable complex. The color was allowed to develop at room temperature for 30 min, and the optical density at 630 nm was measured with a Gilford spectrophotometer (Gilford Instruments, Oberlin, OH). The spectrophotometer was calibrated against 200 µl of water and 800 µl of the malachite green-molybdate-polyvinyl alcohol mixture.

The ADPase pH study used sodium acetate at pH below 6.0, PIPES between pH 6.0 and 7.5, and TRIS above pH 7.5 with 500 µ*M* 1-levamisole added to TRIS to inhibit any alkaline phosphatase activity; MOPS was later substituted for PIPES because it has a pI closer to pH 7.4 and, like PIPES, it neither effects enzyme activity nor binds divalent cations. For inhibitor-activator studies, the subcellular fraction was incubated with the effector and buffer for 2 min at room temperature before Mg^{2+} and substrate were added to the assay.

Electron Microscopy

Subcellular fractions were chemically fixed with 1% glutaraldehyde in Dulbecco's PBS (Polysciences Inc., Warrington, PA), washed with 8% sucrose solution, and postfixed with 2% osmium tetroxide solution buffered with .1 *M* sodium cacodylate solution at pH 7.4. Fixed samples were then dehydrated in a graded ethanol series and embedded in an epoxy resin mixture. Thin sections, cut with diamond knives, were stained with lead citrate and uranyl acetate solutions and examined in a Zeiss (10B electron microscope (Zurich, Switzerland).

For negative staining, 10- to 20-µl aliquots of dilute suspensions of subcellular fractions were absorbed to carbon and Formvar-coated specimen grids (Polysciences) for 1 min, and the grids were washed with 5 to 10 drops of 2% uranyl acetate solution and dried in air.

RESULTS AND DISCUSSION

pH and Activity

In the study of the general acid phosphatase activity of rat mammary gland (14), some ADPase activity was observed to be associated with membrane-derived fractions. Preliminary experiments showed a twofold stimulation by

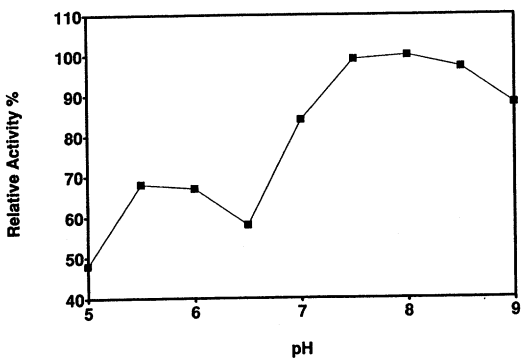

Figure 1. The pH activity curve for Mg^{2+}-stimulated adenosine-5′-diphosphatase (ADPase) in lactating bovine mammary microsomes (■). Average values for three preparations.

Mg^{2+} for the microsomal fraction. In the present study, membrane-associated fractions (Golgi apparatus and microsomes) of lactating bovine mammary gland were studied to determine their pH profiles for Mg^{2+}-stimulated hydrolysis of ADP. An apparent bimodal profile was obtained for the microsomal fraction (Figure 1). Results were similar for the Golgi apparatus. This is in contrast to the relatively broad pH profile reported for UDPase activity in lactating rat mammary gland (12). In fact, previous reports on the nucleotide specificity of membrane-associated NDPase in Golgi apparatus (3, 18, 21) have indicated a distinct lack of activity toward ADP. Activity of ADPase at pH 5.8 and 7.4 appeared to be rather constant for both the microsomal and Golgi apparatus preparations (Tables 1 and 2). Although the pH maximum appeared to be near 8.0, assays for ADPase were conducted at pH 7.4 to avoid alkaline phosphatase activity. In previous works (3, 18, 21), enzyme activity studies of Golgi apparatus NDPase were conducted on detergent extracts; here ADPase was studied on intact Golgi apparatus fraction, which had been frozen and thawed.

Subcellular Distribution of ADPase

Subcellular fractions of lactating bovine mammary gland were isolated by differential centrifugation and assayed for ADPase activity (Table 2). Lactose synthetase, NADPH-cytochrome *c* reductase, and succinate dehydrogenase were used as marker enzymes for Golgi apparatus, microsomes, and mitochondria, respectively. All membrane-associated fractions showed increased specific activity over homogenate for ADP hydrolysis at pH 7.4; the greatest increase occurred for the Golgi apparatus fraction.

An ADPase has been reported to occur as an ectoenzyme on the plasma membranes of a number of tissues such as lung, heart, and smooth muscle (7, 8, 13). In addition, this enzyme, found on the outer surface of erythrocytes, is thought to limit ADP-mediated platelet aggregation (15). Thus, it is not surprising that specific activity would be high in the microsomal fraction of bovine mammary gland. This fraction contains membranes from both secretory epithelial cells as well as myoepithelial cells. In contrast, the Golgi apparatus is characteristic of secretory epithelial cells, and significant ADPase activity has not been reported for this fraction from mammary tissue. One other study (13) suggested that some ADPase activity partially copurified with galactosyl transferase from cultured aortal cells. Note that TPPase activity, a marker of *trans*-Golgi (21), copurifies with lactose synthetase (Table 2). Morphological examination of the Golgi apparatus fraction by electron microscopy showed it to be 85 to 90% pure and relatively free of mitochondria and smaller vesicles. Subcellular fractions were assayed for ADPase at both pH 5.8 and 7.4. The activity toward ADP at pH 7.4 appears to be higher and more concentrated in Golgi apparatus than the activity that occurs at pH 5.8.

Activator and Inhibitor Studies

Phosphohydrolyases can be classified on the basis of responses to selected inhibitors. Both Ca^{2+} or Mg^{2+} can stimulate activity, which also occurs in other tissues (6, 8, 13) (Table 3). The membrane fractions of lactating bovine mammary gland thus contain metal-stimulated ADPase. The degree of metal ion stimulation for Mg^{2+} is similar to that of Ca^{2+}; Mg^{2+} is the more likely physiological activator, except for the Golgi apparatus where the free $[Ca^{2+}]$ is thought to be threefold greater than that of free $[Mg^{2+}]$ (10, 11).

Both Golgi apparatus and microsomal ADPases were only slightly inhibited by oua-

TABLE 3. Effects of selected activators and inhibitors on adenosine-5′-diphosphatase (ADPase) activity in Golgi apparatus and microsomal fractions.[1]

Effector	Concentration	% Activity	
		Golgi apparatus	Microsomal fraction
$MgCl_2$	10 m*M*	100	100
$CaCl_2$[2]	10 m*M*	95	96
No M^{2+}	. . .	65	64
$LaCl_3$[3]	1 m*M*	6	7
$LaCl_3$ + (Mg^{2+})[3]	1 m*M*	49	66
Ouabain	5 m*M*	100	100
Oligomycin	100 μg/ml	86	87
Levamisole	500 μ*M*	94	98
Vanadate	400 μ*M*	38	39
NaF	10 m*M*	8	13
Ap_5A[4]	.1 μ*M*	100	110
App[NH]p[5]	200 μ*M*	65	75

[1]Standard assay pH 7.4.

[2]For Ca^{2+}, this ion was substituted for Mg^{2+}.

[3]For La^{3+}, 1 m*M* was added with and without Mg^{2+}.

[4]P^1, P^5-di(adenosine-5′) pentaphosphate.

[5]5′-Adenylylimidodiphosphate.

bain, oligomycin and levamisole ruling out Na^+/K^+ and F_1F_0 ATPases and alkaline phosphatase, respectively, as responsible for the phosphatase activity observed (Table 3). The ADPase activity is typical of a variety of phosphatase enzymes in that both Golgi apparatus and microsomal fractions are inhibited by vanadate, NaF, and $LaCl_3$. The ADPase activity can arise from the action of adenylate kinase (8, 15), but this latter enzyme generally does not liberate significant inorganic phosphate, the basis for the assay used here. In addition, P^1, P^5-di(adenosine-5′) pentaphosphate is a selective inhibitor of adenylate kinase; at concentrations completely inhibitory to the latter enzyme, P^1, P^5-di(adenosine-5′) pentaphosphate does not inhibit either Golgi apparatus or microsomal ADPase, arguing against the ADPase activity being due to adenylate kinase. The compound adenylylimido diphosphate inhibits membrane-associated ADPases with an inhibition constant of approximately 100 μ*M* (7, 15). At 200 μ*M*, 35% inhibition of ADPase activity was observed for Golgi apparatus and 25% for microsomal fraction of lactating bovine mammary gland. The pattern of inhibitors observed in Table 3 supports the occurrence of an ADPase in lactating bovine mammary gland but with a relatively high background of nonspecific hydrolysis, which could be due to residual milk in the gland.

The results presented herein suggest strongly the existence of a Ca^{2+}/Mg^{2+}-ADPase in lactating bovine mammary gland. Studies on Golgi apparatus from other tissues (3, 18, 21) have documented the presence of UDP-specific enzymes (which are distinct from ADPase) and have been suggested to be possible marker enzymes for Golgi apparatus. In those latter studies, UDPase was detergent extracted. The ADPase activity here has a distinct pH curve, different inhibitor patterns, and was not detergent extracted. A similar enzyme has recently been reported to occur in Golgi apparatus from lactating rat mammary gland (9).

Activity of ADPase has been reported to occur associated with plasma membranes in a variety of tissues and cells (Table 4). Its occurrence in mammary gland microsomes is expected because of the various cell types that

TABLE 4. Comparison of properties of adenosine-5′-phosphatase (ADPase) preparations.

Source	Rat lung	Human	Rat heart	Swine aorta	Bovine mammary
Subcellular localization	Plasma membrane	Red cell membrane	Sarcolemma	Plasma membrane	Golgi and microsomes
Cations	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}	Mg^{2+}/Ca^{2+}
pH	7.5	7.4	7.4	7.3	5.8/8.0
App[NH]p[1]	60% I	. . .	60% I	. . .	30% I
Ap_5A[2]	. . .	10% I	20% I	. . .	0% I
Levamisole	0% I	0% I	0% I	0% I	0% I
References	(7)	(15)	(8)	(13)	This study

[1]5′-Adenylylimidodiphosphate.

[2]P^1, P^5-di(adenosine-5′) pentaphosphate.

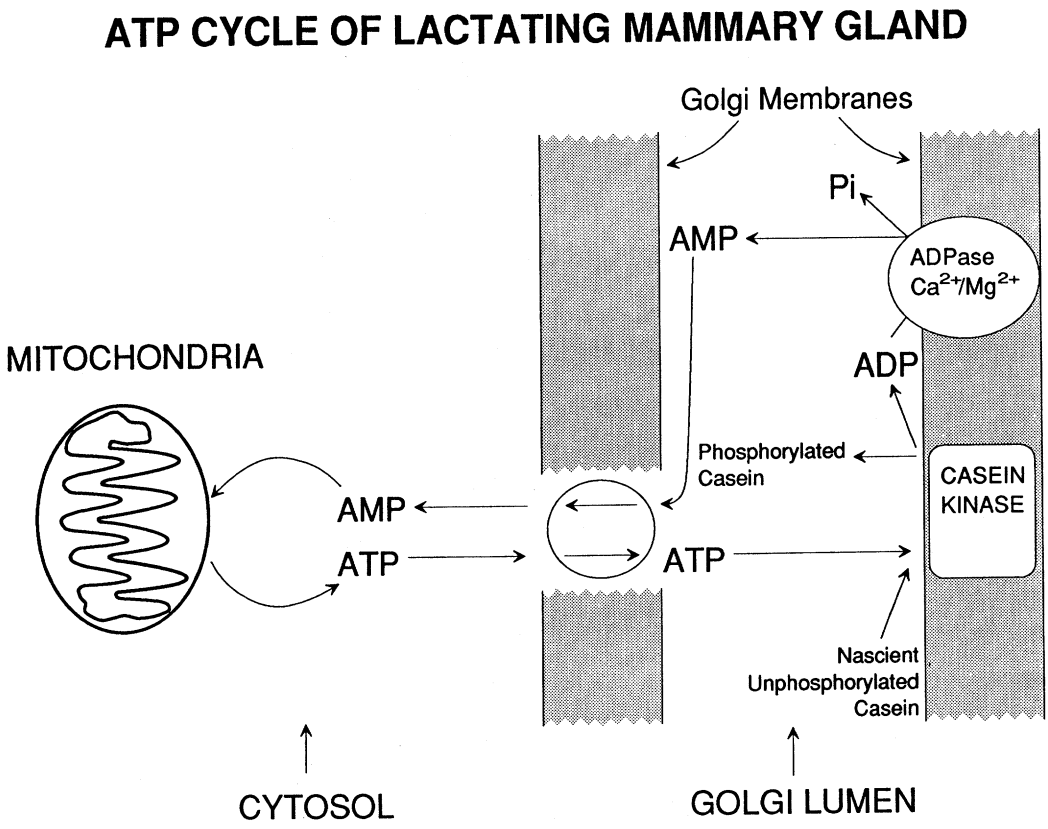

Figure 2. The ATP cycle in lactating mammary gland. The adenosine-5′-diphosphatase (ADPase) described in this manuscript completes the cycle necessary to transport the ATP required to phosphorylate casein in Golgi apparatus. The ATP ↔ AMP transport (4) and casein kinase (1, 22) have previously been described for Golgi apparatus from lactating mammary glands.

occur in the gland (myoepithelial and endothelial cells). However, the Golgi apparatus is a distinctive morphological marker for secretory epithelial cells (14). The occurrence of ADPase in Golgi may be a logical extension of one of the most important functions ascribed to this fraction, namely, phosphorylation of casein (1). The casein kinase, which carries out this function, transfers the γ-phosphate group of ATP to specific serine residues (2). For each residue phosphorylated, an ADP is generated. Because Capasso et al. (4) demonstrated that the nucleotide transport system in rat mammary Golgi is selective for AMP and ATP, there is a need for an ADPase enzyme to convert the diphosphate to monophosphate to complete the ATP cycle depicted in Figure 2. It is interesting to note that the specific activity for casein kinase from bovine mammary Golgi apparatus is 3.10 nmol/min per mg (22). At pH 5.8, the specific activity of ADPase is twice this figure, and at pH 7.4 it is 10-fold greater (Table 2); even considering a moderately high phosphatase background, the ADPase to kinase ratio is high. This indicates that ADP would be rapidly removed and would not cause product inhibition of the casein kinase; apparently, there is sufficient ADPase activity to maintain casein phosphorylation.

Based on comparison of the properties of the mammary enzyme with the known ADPases, it appears (Table 4) that the Golgi apparatus and microsomal fractions do contain a specific ADPase. The pH profile of the ADPase is somewhat unique, and such a profile could allow the enzyme to respond to

either a basic or the more acidic environment found in milk (11) and, presumably, in secretory vesicles (10). Both Ca^{2+} and Mg^{2+} stimulate activity of the enzyme, but Ca^{2+} is more likely important in milk secretion, whereas Mg^{2+} may be related to the plasma membrane-associated enzyme. Thus, both activities may respond to the wide variety of metal ion fluxes in lactating mammary gland.

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